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β-Thalassemia and Hemoglobin Types in Argentina: Determination of Most Frequent Mutations

Néstor W. Soria,[1,2]* Clara L. Tulián,[2] François Plassa,[2] and Germán A. Roth[1]

[1]Departamento de Química Biológica-Centro de Investigaciones en Química Biológica de Córdoba, Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina
[2]Centro Médico Bancario de Córdoba, Córdoba, Argentina
[3]Laboratoire de Génétique Moléculaire, Institut National de la Santé et de la Recherche Médicale U91, Centre Hospitalier Universitaire Henri Mondor, Créteil, France

In order to know the spectrum of β-thalassemia alleles and other mutations affecting the β-globin gene, we analyzed the hemoglobin abnormalities in 24 patients from the Province of Cordoba in Argentina. Molecular screening of samples was performed by the polymerase chain reaction (PCR), using six sets of oligonucleotides to amplify fragments encompassing the whole β-globin coding region and splice junctions, as well as the promoter and 3′ untranslated regions. The altered fragments were determined by denaturing gradient gel electrophoresis (DGGE), and the corresponding mutations were identified by restriction enzyme analysis or by direct sequencing of PCR products. Using this approach, three different β-thalassemia mutations were detected, codon 39 (C→T), IVS-1-110 (G→A), and IVS-1-1 (G→A), and also the hemoglobin S trait. This is the first report of β-thalassemia mutations described in Argentina. Our results show that these mutations are similar to those found in Spain and Italy, possibly due to the important Mediterranean migratory stream received in our country, and could be important for prenatal diagnosis of these diseases in Cordoba, Argentina. Am. J. Hematol. 54:160–163, 1997

Key words: β-thalassemia; β-Thal mutations; β-globin gene; hemoglobin S; framework

INTRODUCTION

Thalassemia syndromes are genetic disorders characterized by absent or deficient synthesis of one or another of the globin chains of hemoglobin. β-thalassemias (β-Thal), due to deficient production of the β-globin chain, are inherited as autosomal-recessive disorders and are distributed primarily among people of Mediterranean, African, and Asian descent [1–3]. Clinical and hematological expression is quite heterogeneous, ranging from severe to intermediate β-Thal or asymptomatic carriers. Restriction endonuclease mapping, cloning, and DNA sequencing have revealed several types of mutations in the β-globin gene that include transcription mutations, RNA-processing mutants, and mutations that affect RNA function. This diverse family of diseases is generally caused by a single base change or short DNA deletions or insertions [4], but a few large deletions affecting part or all the β-globin gene have been identified. Advances in molecular genetic techniques, especially the polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE), have resulted in the elucidation of many of the mutations causing β-Thal [5]. However, each population appears to have its own characteristic set and frequency of β-Thal mutations [6]. Argentina is a cosmopolitan community, with a population of 35 million, comprising different ethnic groups, Italian and Spanish accounting for about 80% of the immigratory flow. At the moment, the only abnormalities described have been Hb Buenos Aires [7], Hb J-Cordoba [8], Hb S [9], and some preliminary characterizations of molecular mutations of

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*Correspondence to: Dr. Néstor W. Soria, Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, C.C. 61-Córdoba 5016, Argentina.

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TABLE I. Hematological Data (Average Values Only) and Types of Mutations

Mutations	Alleles	n[a]	Hb (g/dl)	RBC (10^{12}/l)	MCV	Hb A_2[b] (%)	Hb F (%)
None (normal values)	β/β		13.0	4.50	80–90	0.8 ± 0.2	<3
IVS-1-110 (G→A)	β/β^{+}	14	11.6 ± 1.6	5.71 ± 0.77	67.6 ± 3.3	3.9 ± 1.1	1.7 ± 0.8
Codon 39 (C→T)	β/β^{0}	7	12.3 ± 0.7	6.12 ± 0.49	64.7 ± 1.2	3.1 ± 0.3	2.4 ± 1.8
IVS-1-1 (G→A)	β/β^{0}	1	12.3	6.26	65	4.7	1.6
β^{S}	β^{S}/β^{S}	1	8.1	2.43	85	1.2	6.3
β^{S}/codon 39 (C→T)	β^{S}/β^{0}	1	9.2	3.20	82	1.0	7.0

[a]Number of subjects. Results represent the mean of all studied patients in each group, and the respective standard deviation of these means.
[b]Hb A_2 was determined by cellulose acetate pH 8.4 electrophoresis and subsequent scanning densitometry.

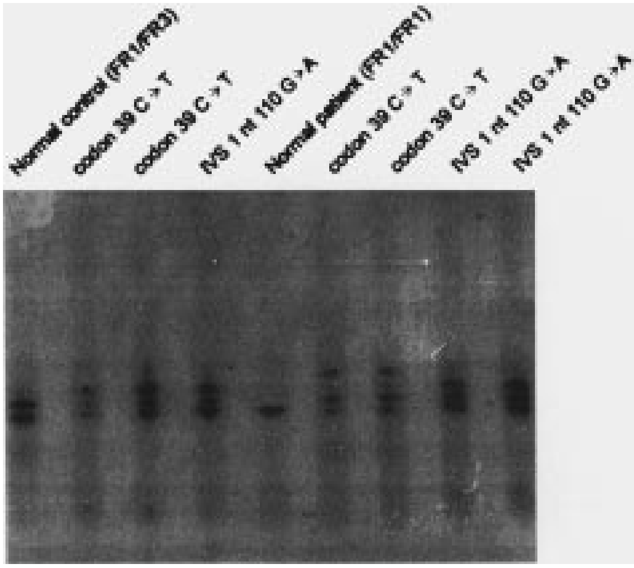

Fig. 1. Detection by DGGE analysis of fragment C of β-globin exon 2. Fragments were subjected to electrophoresis in a 30–80% denaturing gradient gel at 10 V/cm for 5 hr. The gel was stained with ethidium bromide and visualized under ultraviolet light. Normal control is a Framework 1/3 heterozygote for the IVS-2-16 (C→G) polymorphism. Any heteroduplexed pattern different from this control indicates the presence of a mutant allele. The samples analyzed were all heterozygous for the corresponding mutation indicated in each line, except that lane 5 that corresponded to a normal patient. All alleles displaying altered melting behavior were then characterized by restriction endonuclease digestion.

the β-globin gene [9–11]. We analyzed the β-globin genes of 24 unrelated patients with hematological problems. The information gained from this study will permit us to know the mutations and their frequencies in the analyzed population.

MATERIALS AND METHODS

Twenty-two patients with β-Thal and 2 with other hematological alterations who received treatment at the Centro Medico Bancario de Cordoba were selected on the basis of their hematological features (Table I). Informed consent was obtained. Blood samples were collected onto EDTA as anticoagulant, and hematological data were collected with an automated cell counter. Red cell lysates were studied by electrophoresis on agar-citrate, pH 6.2, and cellulose acetate, pH 8.4, and by isoelectrofocusing that provided quantitative data for HbA_2 and Hb F [12]. It is worthy of note that our determinations of Hb A_2 by cellulose acetate pH 8.4 electrophoresis and subsequent scanning densitometry yielded reproducible but lower values (Table I) than those usually described.

DNA was isolated from leukocytes by phenolchloroform extraction [13], and purified by the method of McInnes et al. [14]. The nucleotide sequences of the primers for amplification of each fragment were those described by Ghanem et al. [5]. PCR was carried out in a Perkin Elmer Cetus (Foster City, CA) thermal cycler in a final volume of 100 μl containing 0.2 μg of DNA sample, 0.2 μmol of each oligonucleotide, 100 μM of each dNTP, and 0.5 units of *Taq* DNA polymerase (Promega) in the supplied reaction buffer. The cycling conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 40 cycles, followed by a 10-min extension at 72°C. One set of oligonucleotides (IVS-1-110 and PCO14b) was used to identify the mutation IVS-1-110 (G→A). To detect this mutation, PCR products with an artificial base substitution at IVS-1-112 were generated by the primers IVS-1-110 (5′-CGGATAAAAGGG-TGGGAATCCGACGAC-3′) and PCO14b (5′-ACTCC-CAGGAGCAGGGAGGG-3′). Amplified DNA from the normal alleles presents an additional GGCC site for the restriction enzyme *Hae*III, that it is not created in the mutated allele. For those mutations that alter a restriction enzyme recognition site, restriction endonuclease analysis was applied. The enzymes *Mae*I and *Cvn*I were used for detection of the following mutations, codon 39 (C→T) and β^{s}, respectively [15]. Digestion conditions were those indicated by the enzyme manufacturers.

For DGGE analysis, the gel apparatus has been described elsewhere [1]. Each amplified DNA sample (15 μl) was subjected to electrophoresis at 160 V in 6.5% polyacrylamide gel containing a linearly increasing denaturant of 7 M urea and 40% formamide. The electropho-

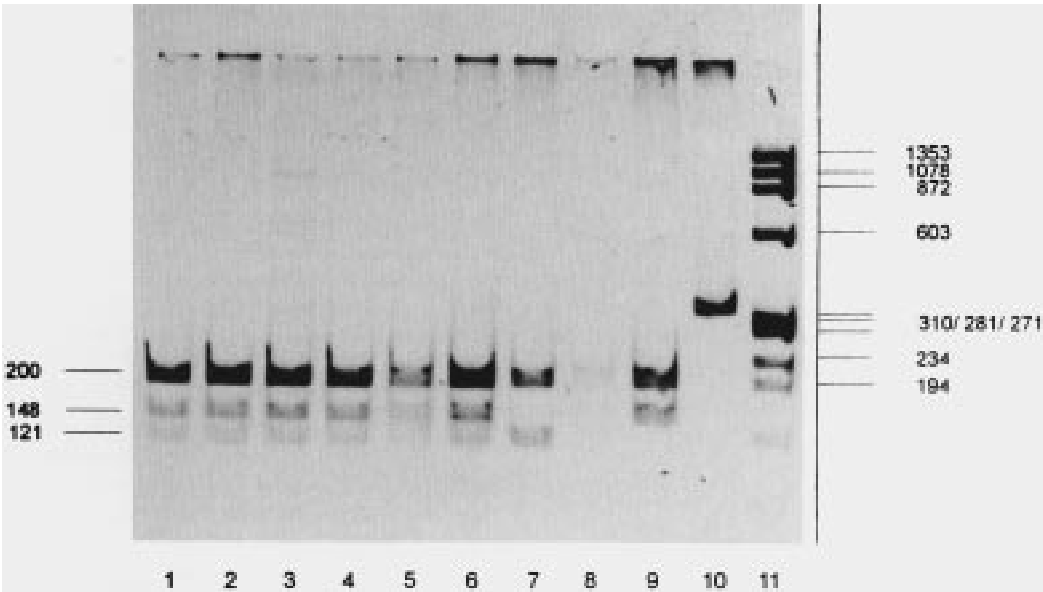

Fig. 2. Detection of mutation IVS-1-110 (G→A) by polyacrylamide gel electrophoresis. The 348-base pair (bp) PCR product from the normal chromosome was digested with the restriction enzyme *Hae*III into 200-, 121- and 27-bp fragments, while 200- and 148-bp fragments were generated from chromosomes with the IVS-1-110 mutation. Only the 200-, 148-, and 121-bp bands were visible on electrophoresis under the conditions used. Lanes 1–6, heterozygous individuals for the IVS-1-110 (G→A) mutation; lane 7, individual without the IVS-1-110 (G→A) mutation; lane 9, individual homozygote for the IVS-1-110 (G→A) mutation; lane 10, undigested amplified DNA (348 bp); lane 11, molecular weight marker $\phi \times 174$ cut with *Hae*III.

retic conditions and the routine for each fragment were according to Ghanem et al. [5]. Direct genomic sequencing by asymmetric amplification [17] was performed to identify the mutation IVS-1-1 (G→A) after purifying the PCR product with a QiaGen kit, (Chatsworth, CA) and using the PCR oligonucleotides as sequencing primers.

RESULTS

The hematological data are in Table I. The diagnostic criteria for selection of the 22 analyzed β-Thal patients were MCV <77 fl, Hb A_2 >2.5%, and exclusion of iron deficiency. The other 2 patients showed anemia with a concomitant abnormal pattern of hemoglobins by electrophoretic analysis. We observed three different β-Thal mutations in the patients studied. All subjects were heterozygous for the thalassemic allele, except one who was β^s/β^0, and another was homozygous for β^s. The mobilities obtained by DGGE of fragment C for some patients are shown in Figure 1. Samples from the patients with abnormal mobilities in this fragment were subjected to restriction endonuclease analysis to identify the mutations IVS-1-110 (G→A) (Fig. 2) and codon 39 (C→T). Alterations localized in fragment B, such as IVS-1-1 (G→A) and β^s, were determined in similar form (data not shown). No alterations were observed in the other fragments studied, encompassing the whole β-globin gene.

In the 26 altered alleles studied, the two commonest mutations were IVS-1-110 (G→A), accounting for 53.8%, and codon 39 (C→T), accounting for 30.8%. Other mutations were detected in moderate frequency, i.e., IVS-1-1 (G→A) in 3.8% and β^s in 11.6%.

DISCUSSION

This study sought to detect and characterize β-globin gene abnormalities in Argentinean patients, and has resulted in the identification of three different β-Thal mutations (IVS-1-1 (G→A), IVS-1-110 (G→A), and CD 39 (C→T) and the hemoglobin S trait. These three β-Thal mutations have been found in several Mediterranean populations, accounting for >70% of the β-Thal chromosomes [6]. In particular, studies in Sapin and Italy showed that they were the three most common mutations, representing 76% and 70% of all β-Thal mutations, respectively [3]. A preliminary study done by Brandariz et al. [11] in patients from Buenos Aires, Argentina showed eight different β-Thal mutations. Among them, IVS-1-1 (G→A), IVS-1-110 (G→A), and CD 39 (C→T) were present at high frequency, accounting for 64% of β-globin gene defects; meanwhile, the mutations IVS-1-6 (T→C), IVS-2-1 (G→A), frameshift CD 6 (−A), frameshift CD 5 (−CT), and CD 15 (G→A) were found at low frequency. These data, in addition to the results reported in this paper, show that the types and frequencies of β-Thal mutations in the two most populated areas of Argentina

are similar to those observed in Spain, Italy, and other European countries, probably due to the historical migration of different ethnic groups, especially from Europe. Information on the frequency of different β-Thal mutations in the Argentinean population is crucial for the organization of prenatal diagnosis by DNA analysis. These programs have had notable success, and recent reports indicate that the incidence of live births with β-Thal major in parts of Greece and Italy is 10–20% the rate expected in the absence of the prevention programs [18]. Although several methodologies can be applied for screening known mutations [19], DGGE is an easy and rapid methodology, useful for detecting known as well as unknown mutations.

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REFERENCES

1. Weatherall DJ, Clegg JB: "The Thalassemia Syndromes." Oxford: Blackwell Scientific Publications, 1981.
2. Kazazian HH, Boehm CD: Molecular basis and prenatal diagnosis of β-thalassemia. Blood 72:1107, 1988.
3. Cao A, Pirastu M, Goossens M: β thalassaemia mutations in Mediterranean populations. Br J Haematol 71:309, 1989.
4. Orkin SH, Kazazian HH, Antonarakis SE, Goff SC, Boehm CD, Sexton JP, Waber PG, Giardina PJV: Linkage of β-thalassemia mutations and β-globin gene polymorphisms with DNA polymorphisms in human β-globin gene cluster. Nature 296:627, 1982.
5. Ghanem N, Girodon E, Vidaud M, Martin J, Fanem P, Plassa F, Goossens M: A comprehensive scanning method for rapid detection of β-globin gene mutations and polymorphisms. Hum Mutat 1:229, 1992.
6. Giambona A, Lo Gioco P, Marino M, Abate I, Di Marzo R, Renda M, Di Trapani F, Messana F, Siciliano S, Rigano P, Chehab F, Kazazian HH, Maggio A: The great heterogeneity of thalassemia molecular defects in Sicily. Hum Genet 95:526, 1995.
7. De Weinstein BI, White JM, Wiltshire BG, Lehmann H: A new unstable haemoglobin: Hb Buenos Aires β85 (F1) Phe→Ser. Acta Haematol (Basel) 50:357, 1973.
8. Bardakjian J, Kister J, Rhoda MD, Marden M, Arous N, De Leon J, North ML, Lacombe C, Blouquit Y, Castrane C, Riou J, Rosa J, Galacteros F: Hb J-Cordoba [$\alpha_2\beta_2$95 (FG2) Lys→Met], a new Hb variant found in Argentina. Hemoglobin 12:1, 1988.
9. Soria NW, Roth GA: First case of hemoglobin and β^0-thalassemia detected in an Argentinean girl. Hemoglobin 20:125, 1996.
10. Soria NW, Roth GA: Caracterizacion parcial de una variante de hemoglobina. In: "Proceedings of the XXVIII Reunion Anual de la Sociedad Argentina de Investigacion Bioquımica." Cordoba, Argentina, 1992, p 233.
11. Brandariz S, Arteman P, Levin MJ, Iparraguirre B, Erramouspe B, Timpanaro J, Chiappe G, Beldjord C: Caracterizacion molecular de mutaciones del gen de la β-globina en pacientes talasemicos argentinos. In: "Proceedings of the XXX Reunion Anual de la Sociedad Argentina de Investigacion Bioquımica." Misiones, Argentina, 1994, p 371.
12. Kutlar A, Huisman THJ: Detection of hemoglobinopathies. In Hommes FA (ed): "Techniques in Diagnostic Human Biochemical Genetics." New York: Wiley-Liss, 1991, p 519.
13. Alexander D, Dudin G: Five related Lebanese individuals with high plasma lisosomal hydroxylases: A new defect in mannose-6-phosphate receptor recognition. Am J Hum Genet 36:1001, 1984.
14. McInnes B, Potier M, Wakamatsu N, Melancon S, Klavins M, Tsuji S, Mahuran D: An unusual splicing mutation in the HEXB gene is associated with dramatically different phenotypes in patients from different racial backgrounds. J Clin Invest 90:306, 1992.
15. Kulozik AE, Lyons J, Kohne E, Bartram CR, Kleinhaner E: Rapid and non-radioactive prenatal diagnosis of β-thalassemia and sickle cell disease: Application of the polymerase chain reaction (PCR). Br J Haematol 70:455, 1988.
16. Attree O, Vidaud D, Vidaud M, Amselem S, Lavergue J, Goossens M: Mutations in the catalytic domain of human coagulation factor IX: Rapid characterization by direct genomic sequencing of DNA fragments displaying an altered melting behavior. Genomics 4:266, 1989.
17. Gyllestein UB, Erlich HA: Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQ α locus. Proc Natl Acad Sci USA 85:7652, 1988.
18. Cao A, Rosatelli MC, Galanello R: Population-based genetic screening. Curr Opin Genet Dev 1:48, 1991.
19. Embury SH: Advances in the prenatal and molecular diagnosis of the hemoglobinopathies and thalassemias. Hemoglobin 19:237, 1995.